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Genetics

Retroviral-mediated gene transfer into hepatocytes *in vivo*

(retroviral vectors/liver perfusion/nuclear β-galactosidase/gene therapy)

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ABSTRACT **Stable gene transfer into hepatocytes might be used to compensate for a genetic deficiency affecting liver function or to deliver diffusible factors into the blood stream. In rats, we have combined retroviral-mediated gene transfer with a surgical procedure in which the liver is temporarily excluded from the circulation and infected *in vivo*. Partial hepatectomy was performed 24–48 hr before perfusion with virus to induce hepatocyte division and facilitate viral integration. A helper-free recombinant retrovirus coding for β-galactosidase with nuclear localization was used to score cells that expressed the transgene. For at least 3 months after gene transfer, up to 5% of hepatocytes expressed nuclear β-galactosidase. Whereas *in vitro* reimplantation of genetically modified hepatocytes has proved to be inefficient in stably transferring genes into the liver, our approach provides a feasible alternative.**

A method for safe, efficient, and stable introduction of foreign DNA into hepatocytes would allow the development of protocols for the genetic treatment of many inborn errors of the metabolism.

Replication-defective retroviral vectors ensure genomic integration of a few transgene copies in an unrearranged and permanent configuration transmitted to the cell progeny. The stability and long-term expression of transgenes introduced by retroviral vectors *in vivo* has been documented in a variety of somatic tissues (1–4). Use of helper-free packaging cell lines (5) has shown that the replication-defective recombinant retrovirus is not transmitted to nontargeted organs (4, 6) and *a fortiori* to other organisms.

Freshly explanted hepatocytes are susceptible to retrovirus infection (7), and strategies for gene transfer into the liver involving *in vitro* infection of cultured liver cells followed by reimplantation have been proposed. However, whereas fresh hepatocytes can be efficiently engrafted into the spleen (8, 9) or the liver (10), most *in vitro* cultured and infected cells lose their capacity to be transplanted back (11). Therefore, the feasibility of large-scale gene transfer to the liver by this approach is unlikely. Our purpose was to develop an alternative method whereby the hepatocytes would directly receive the transgene *in situ*. Because cell division is required for genomic integration of retroviruses and because most hepatocytes are quiescent cells in the adult liver, retroviral infection of the steady-state organ is expected to be inefficient. On the other hand, partial resection of the liver is followed by a regenerative phase that rapidly reconstitutes a full-size organ. Many hepatocytes enter the cell cycle during this process (12, 13) and should be susceptible to retrovirus infection at this time. After complete restoration of the organ weight, hepatocytes return to a quiescent state (12, 13), and prolonged persistence of infected cells may be expected. Therefore, the *in situ* infection of hepatocytes with recombinant retroviral vectors may be a suitable method for long-term complementation of a genetic defect.

To target *in vivo* retrovirus infection to the liver, we surgically excluded the remnant liver lobes of partially hepatectomized rats from the circulation. A 10-min asanguineous perfusion of the regenerating tissue was then performed with a solution containing purely defective retrovirus vectors. After blood circulation was reestablished, liver regeneration proceeded until completion. A nuclear β-galactosidase reporter gene (14) encoded by the vector allowed the detection of infected cells in animals sacrificed 2–14 weeks after the perfusion. The expression of the recombinant provirus was observed in up to 5% of the hepatocytes, whereas in other tissues, including spleen, lung, kidney, and brain, no recombinant provirus could be detected in cellular DNA by using the polymerase chain reaction (PCR).

MATERIALS AND METHODS

Amphotropic Retroviral Vector and Virus Assays. The pMFG retroviral vector was obtained from B. C. Guild, P. Robbins, L. Cohen, and R. C. Mulligan (Whitehead Institute, Cambridge, MA). A 3.5-kilobase pair (kbp) DNA fragment containing a modified *lacZ* gene (*nls-lacZ*), which codes for the *Escherichia coli* β-galactosidase fused to a 21-amino acid nuclear-localization sequence from simian virus 40 large tumor antigen (14), was introduced at the *Bam*HI site. The *nls-lacZ* gene was transcribed from the long terminal repeat (LTR), and the mRNA was spliced by using the viral donor and acceptor sites. Sequences in the Moloney murine leukemia virus *gag* gene (up to position 1035) have been retained to increase the packaging efficiency of the unspliced transcript. The pMFG-NB construct was transfected into the ΨCRE ecotropic packaging cell line (5) by calcium phosphate–DNA coprecipitation. Forty-eight hours later, the culture medium was harvested and used to infect an amphotropic packaging cell line, ΨCRIP (5). Individual ΨCRIP clones expressing the *nls-lacZ* gene were isolated after fluorescence-activated cell sorting of the infected population with fluorescein di-β-D-galactopyranoside as described (15). Titers of *nls-lacZ*-transducing retroviruses were determined by infecting mouse NIH 3T3 fibroblasts with serial dilutions of the culture medium from each clone. Forty-eight hours later, the cells were incubated with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal; Sigma) substrate at 32°C for 8 hr to reveal nuclear β-galactosidase activity (16), and cell clusters with blue nuclei were counted. Filtered supernatant from the NB5 clone used in this study contained 5×10^5 β-galactosidase focus-forming units (ffu) per ml. The absence of contamination with replication-competent retrovirus was controlled in the MFG-NB retrovirus vector stocks, in animal sera, and in liver and spleen extracts by an amplification and mobilization assay whose sensitivity is <1 ffu of ecotropic or

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Abbreviations: LTR, long terminal repeat; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; nls, nuclear localization sequence; ffu, focus-forming units.

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amphotropic helper virus per ml. This assay is based on the rescue of a defective retroviral genome containing *lacZ* and *neo* genes (17). The MFG-NB virus stocks as well as all of the animal samples tested were negative.

Primary Culture of Rat Hepatocytes. Single-cell suspensions of hepatocytes were prepared from young adult Lewis rats by perfusing the liver with a collagenase solution (0.4 mg/ml, Worthington). Cells were plated onto plastic dishes (10^5 per cm^2) in Ham's F-12 medium containing 0.1% bovine serum albumin, 5 μg of bovine insulin per ml, 1 μM hydrocortisone hemisuccinate, and 10% (vol/vol) fetal calf serum. After 5 hr, hepatocyte monolayers were refed with the same medium in the absence of serum. Cultures were infected 3 days later by overnight incubation with filtered medium from the NB5 clone and 8 μg of polybrene per ml. Cells were fixed on day 6 with 0.5% glutaraldehyde and 1% formaldehyde in phosphate-buffered saline (PBS) and stained for β-galactosidase activity.

Hepatectomy and Asanguineous Surgical Perfusion of the Liver. A two-thirds hepatectomy was performed as described by Higgins and Anderson (18) by resecting the left lateral and median liver lobes. The right adrenal vein was ligated during this operation. Twenty-four hours or 48 hr later the remaining right lateral and caudal liver lobes were isolated from the blood flow and perfused as described by Adam *et al.* (19) with minor modifications. After ligation of the gastroduodenal vein, blood supply to the liver was interrupted by successively clamping the hepatic artery, the portal vein, the infrahepatic vena cava, and the suprahepatic vena cava. Culture supernatant was harvested from NB5 cells, filtered, added with 8 μg of Polybrene per ml, heated at 37°C, and infused through a cannula inserted into the portal vein with a flow rate of 5 ml/min. Perfusion medium was collected from the infrahepatic vena cava and not reinjected. After a 10-min perfusion, hepatic blood flow was reestablished. The operations were performed on deeply ether-anesthetized animals. All treated rats survived surgery.

Liver Sections and Histochemical Staining. The liver was fixed *in situ* by perfusing a 4% paraformaldehyde solution. After removal, the organ was cut into 5-mm-thick blocks. Blocks were either incubated overnight at 32°C with X-Gal substrate or immersed overnight in PBS with 30% sucrose at 4°C, frozen in isopentane, and used to prepare 8- to 10-μm-thick cryostat sections. Detection of albumin and detection of a sinusoid membrane antigen specific for the hepatocyte (20) were performed after X-Gal staining of the slides. Normal goat serum or goat antiserum directed against rat albumin (Nordic, Lausanne, Switzerland) was incubated at a dilution of 1:20 on liver sections previously permeabilized with Tween 20 (0.01%) and saponine (0.005%). Rat anti-goat IgG Fab' fragments coupled to peroxidase were used for staining. Mouse monoclonal antibodies directed against a rat hepatocyte sinusoidal membrane antigen or against human colony-stimulating factor 1 as a control were diluted 1:50 and incubated on liver sections in PBS with 0.5% low-fat milk. After the sections were washed, complexes were identified by using rabbit anti-mouse antibodies coupled to biotin and streptavidin.

PCR. PCRs were performed on high molecular weight DNAs isolated from liver, lung, brain, spleen, and kidney of experimental animals. Synthetic oligodeoxynucleotide primers complementary to the DNA sequences located in the *nls-lacZ* gene (5'-CGACTCCTGGAGCCCGTCAGTATC-3') and in the vector upstream of the 3' LTR (5'-GACCACTGATATCCTGTCTTTAAC-3') were used. Forty cycles of amplification by the *Thermus aquaticus* (*Taq*) polymerase (Cetus) were performed in a Hybaid thermoreactor under conditions recommended by the manufacturer—at temperatures of 95°C for denaturation, 55°C for hybridization, and 72°C for elongation. In positive samples, the primers generated a 400-bp fragment that specifically annealed to ^{32}P-labeled *nls-lacZ* probes when analyzed by Southern blot. This PCR assay could detect 1 positive cell in 10^4 cells. In the absence of specific signal, the technique ensured that <200 proviral genomes were present per 2×10^6 cells.

RESULTS

***In Vitro* Infection of Rat Hepatocytes with Amphotropic Retrovirus Vector.** We used a reporter gene to evaluate the possibility of stably transferring genes into hepatocytes with retroviral vectors. A modified *E. coli lacZ* gene encoding β-galactosidase and containing a nuclear localization signal (*nls-lacZ*) (14) was inserted into a Moloney murine leukemia virus-based vector. The nuclear localization of the enzyme permits a precise determination of cell types involved in gene transfer since other cytoplasmic markers can be assayed concurrently (21). In addition, its activity is unambiguously distinguished from any endogenous lysosomal signal. After introduction of the construct into an amphotropic packaging cell line, clones producing high titers of recombinant retroviruses were isolated and shown to be free of replicating helper virus (see *Materials and Methods*). We used primary cultures of rat liver cells to show that supernatants from these producer clones actually infect hepatocytes. Supernatant from clone NB5, which contained 5×10^5 β-galactosidase ffu/ml when assayed on NIH 3T3 cells, infected 15% of cultured liver cells. These cells were mostly hepatocytes, as shown by the histochemical detection of glucose-6-phosphatase in their cytoplasm (data not shown).

When injected into the spleen, freshly explanted rat hepatocytes generate liver tissue, both *in situ* (8, 9) and after migration into the liver (10). We injected either freshly isolated or *in vitro* infected and *nls-lacZ*-marked hepatocytes into the spleens of syngeneic rats. Two weeks later, the animals were sacrificed, and their spleens were examined by histological sections. Whereas splenic liver cell foci were routinely observed after transplantation of freshly explanted cells, none could be obtained when liver cells had been previously cultivated, even when hepatectomy was performed at the time of implantation (data not shown).

***In Situ* Infection of Hepatocytes with Retrovirus Vector in the Regenerating Liver.** We reasoned that a partial hepatectomy followed by the direct injection of a defective retrovirus vector into the remnant liver lobes might bypass the difficulty of reimplanting *in vitro* infected hepatocytes. To prevent rapid dissemination of viral particles in the blood stream and destruction of retrovirus particles by serum complement, we surgically isolated the regenerating liver from the circulation (Fig. 1). This procedure allowed a prolonged perfusion of the organ and facilitated the infection of target cells in the sinusoid capillaries.

Hepatectomies of two-thirds of the liver mass were performed on adult male Lewis rats (200–250 g) by resectioning the median and left lateral lobes (18). One or 2 days later, the remaining two lobes were excluded from the blood flow by clamping the hepatic artery, the portal vein, and the suprahepatic and infrahepatic venae cavae. Siliconized cannula were introduced into the portal vein and infrahepatic vena cava. Retrovirus-containing medium was perfused for 10 min, and normal blood flow was reestablished after 20 min. Animals were sacrificed 15–100 days later, and their livers were fixed *in situ* by paraformaldehyde perfusion. Five-millimeter-thick sections and cryostat sections were examined for the presence of cells exhibiting nuclear β-galactosidase activity.

Nuclear β-galactosidase activity could be readily detected on liver blocks in the animals with the greatest activity (rats 8 and 9, Table 1) (Fig. 2 *A* and *B*). In all animals, the presence of β-galactosidase-positive cells was detected on X-Gal-stained cryostat sections (Fig. 2*C*). Table 1 shows the num-

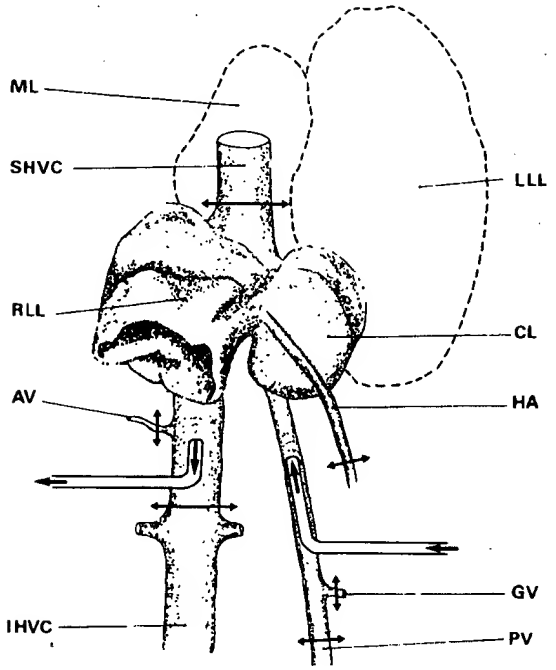

FIG. 1. Selective perfusion of remnant rat liver lobes after partial hepatectomy. The resected liver lobes appear as dotted lines. Double-headed arrows show the position of ligatures and clamping. The direction of the perfusion flow is indicated with arrows. LLL, left lateral lobe; ML, median lobe; RLL, right liver lobe; CL, caudal lobe; AV, right adrenal vein; HA, hepatic artery; PV, portal vein; GV, gastroduodenal vein; IHVC, infrahepatic vena cava; SHVC, suprahepatic vena cava.

bers of positive cells scored on sections chosen randomly from the hepatic parenchyma of experimental animals. The fraction of infected cells in the whole organ was estimated to be 1–5% (20–80 × 10^6 cells) in rats 4–10, which had been perfused 24 hr after partial hepatectomy. In contrast, <0.05% of the cells were found to be infected in rats 1, 2, and 3, which were perfused 48 hr after partial hepatectomy. The number of infected cells in rat 10, which was sacrificed 14 weeks after treatment, was as high as in rats 4, 5, 7, and 6 sacrificed respectively 3, 3, 2, and 6 weeks after the perfusion. β-Galactosidase-positive cells appeared in small clusters (Table 1) and were observed scattered throughout the hepatic parenchyma (Fig. 2*A*). These clusters contained an average number of 1.7 cells, suggesting that most of the cells underwent only one division after the integration of the retrovirus vector genome. No positive cells were observed in either portal or terminal hepatic vascular walls.

Table 1. Number of *nls-lacZ*-positive cells in the livers of rats after a partial hepatectomy followed by a selective perfusion of the remnant lobes with the MFG-NB amphotropic retroviral vector

Rat	Hours between hepatectomy and perfusion	β-Gal-positive nuclei;* no. per cm^2	Nuclei per cluster†	Days between perfusion and sacrifice
1	48	12.5 ± 1.5	1.19	15
2	48	21.3 ± 11	1.16	21
3	48	31.6 ± 10	1.25	21
4	24	160 ± 85	1.32	21
5	24	260 ± 34	1.55	21
6	24	239 ± 86	2.07	43
7	24	210 ± 30	1.45	15
8	24	570 ± 150	1.78	15
9	24	>1500	ND	15
10	24	297 ± 54	ND	100

ND, not determined; β-Gal, β-galactosidase.
*Data are the mean numbers of nuclei expressing β-galactosidase activity per cm^2 of liver section ± SD. Positive cells were scored on different sections representing a total area of 10–30 cm^2.
†Data are the ratios of the mean number of positive nuclei per cm^2 by the mean number of clusters per cm^2.

Cells expressing the transgene had a characteristically large and frequently double nucleus and were localized in the hepatic columns (Fig. 2*C*). β-Galactosidase-positive liver sections were counterstained with a monoclonal antibody recognizing a specific antigen on the sinusoidal plasma membrane (Fig. 2*D*) (20) or with an antiserum directed against rat albumin (Fig. 2*E*). Immunostaining detection of rat albumin was positive in 91% of liver cells in animals 1 and 4. Cells expressing β-galactosidase in their nuclei were also found to be positive for albumin immunostaining in their cytoplasm (41 of 41 examined cells in rat 1 and 31 of 33 in rat 4). These data showed that β-galactosidase-positive cells were differentiated hepatocytes.

Since the regenerating liver was isolated from the blood flow during perfusion of defective retrovirus vector, we expected that the infection would be restricted to this organ. To ensure that no virus had spread out to other organs, we used the PCR to look for the presence of provirus copies in high molecular weight DNAs prepared from lung, spleen, kidney, and brain as well as from liver (see *Materials and Methods*). Fig. 3 shows that the vector sequences were present in liver cell DNA but absent in DNAs prepared from other tissues. This indicated that the perfusion procedure described here did not result in any significant leak of recombinant retrovirus to other organs. It also suggested that replication-competent helper virus was absent from the stocks used for perfusion and did not appear in the animals as a consequence of the treatment. This latter point was confirmed by using a highly sensitive virus mobilization assay (17) that detected no replicating virus particles in the sera, spleen extracts, and liver extracts of treated animals (data not shown).

DISCUSSION

We show in this paper that retroviral vectors can be used to introduce foreign genes into hepatocytes of the regenerating liver without infecting other tissues. This was obtained by performing a two-thirds hepatectomy followed 24 or 48 hr later by a perfusion of the remnant liver lobes with a helper-free retrovirus vector preparation.

Cells expressing the β-galactosidase transgene were identified in small, scattered clusters. This suggested that most of those cycling hepatocytes that integrated the vector underwent less than two subsequent cell divisions. Because the duration of the adult rat hepatocyte cell cycle is 33 hr (13) and the regeneration process generally proceeds for about 7 days (12, 22), our observation supports the notion that most hepatocytes participate in regeneration. The numbers of infected hepatocytes were higher when the virus perfusions were performed during the peak of DNA synthesis at 24 hr. At this time, the fraction of cells undergoing DNA synthesis is estimated to be 15% (12), and 30% of the hepatocytes enter the cell cycle during the next 12 hr (13). Among this cycling hepatocyte population that is permissive for retrovirus-mediated gene transfer, our procedure allows infection of 5–20% of the cells. This shows that under experimental conditions in which propagation of the infection by a replication competent helper virus is excluded, a highly efficient single-hit infection can affect a significant population of cycling hepatocytes. Since the efficiency of this technique could theoretically be improved, we are currently investigat-

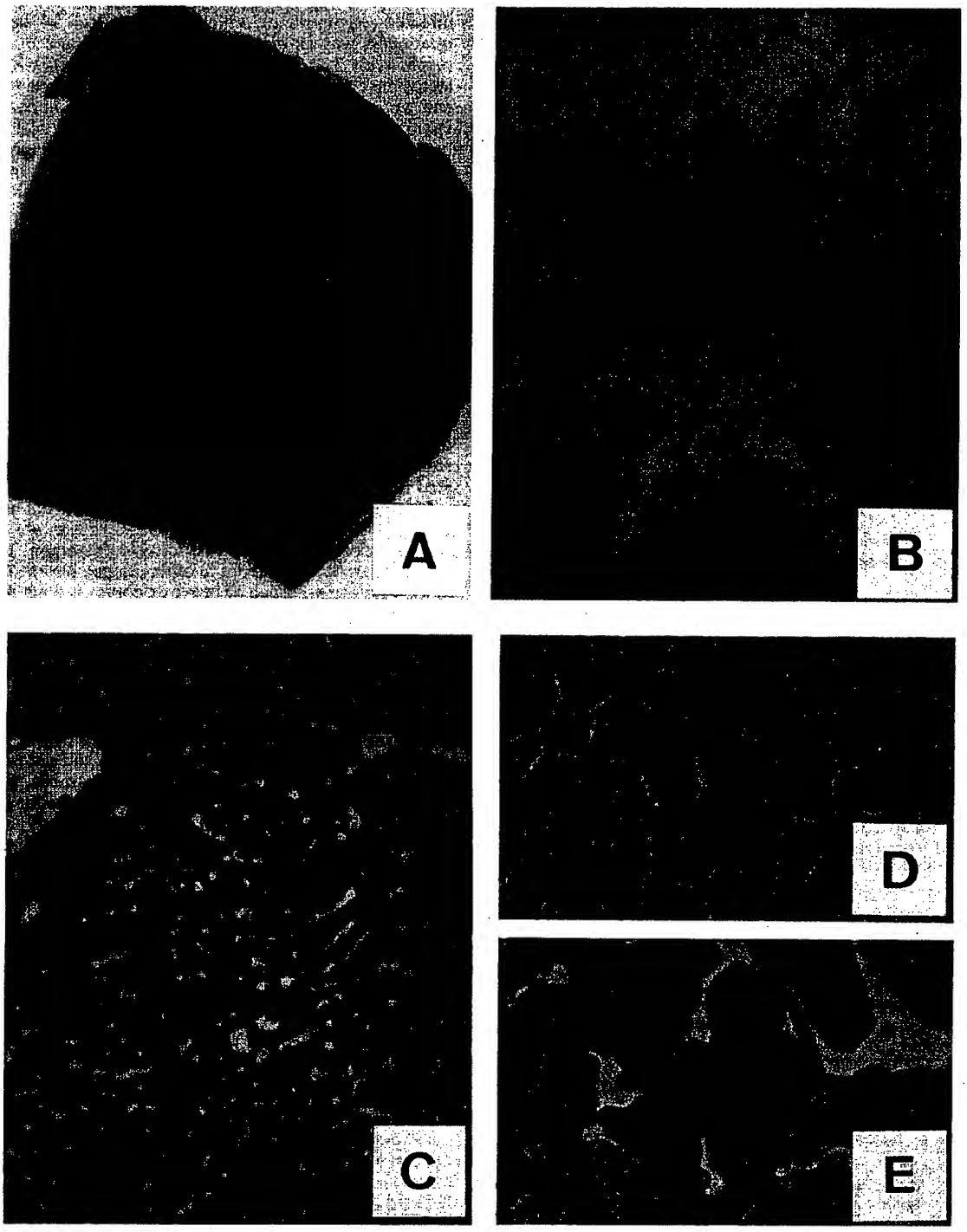

FIG. 2. Histochemical staining of livers from treated animals. (*A* and *B*) X-Gal staining of 5-mm-thick liver sections from rat 9 examined with a binocular microscope (*A*, ×7.5; *B*, ×45.) (*C*, *D*, and *E*) Representative cryostat sections stained with X-Gal and counterstained respectively with hematoxylin and eosin (*C*), a monoclonal antibody directed against a sinusoid membrane antigen specific of the hepatocyte (20) (*D*), and a goat anti-serum directed against rat albumin (*E*). (*C–E*, ×375.)

ing the effects of more concentrated virus stocks, increased perfusion times, and addition of hepatocyte growth factors (23, 24) to the perfusion medium.

Most gene-transfer protocols to somatic tissues using retrovirus vectors involve the explantation of target cells, followed by their *in vitro* infection and reimplantation into the organism. In agreement with recently published work (11), we have observed that cultured hepatocytes rapidly lose most of their *in vivo* repopulating capacities, negating the possibility of using them for large-scale organ reconstitution. We show here that the *in situ* approach provides an alternative for gene transfer into the hepatocytes. *In situ* gene transfer to the

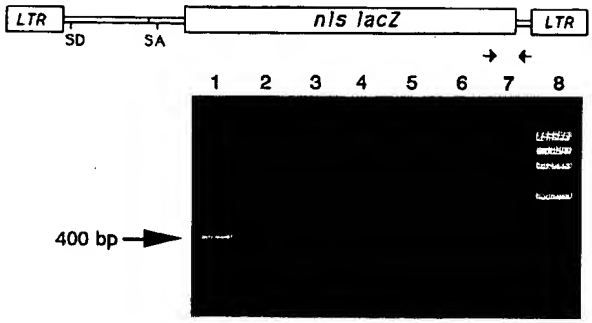

FIG. 3. PCR analysis of the MFG-NB provirus in the tissues of treated animals. (*Upper*) The structure of the MFG-NB retroviral vector is shown. Localizations of the oligodeoxynucleotide primers used for PCR are indicated by arrows. (*Lower*) PCR was performed with synthetic oligodeoxynucleotide primers and 1 μg of high molecular weight DNA. The reaction products were run on 3% agarose gels and revealed by ethidium bromide. DNA samples were prepared from NIH 3T3 cells containing one copy of integrated NB5 genome (lane 1), liver (lane 3), spleen (lane 4), lung (lane 5), brain (lane 6), and kidney (lane 7) of rat 4. Lane 2 shows a control reaction performed in the absence of added DNA, and lane 8 contains molecular weight markers (ϕX174 DNA digested with *Hae* III). After transfer to nylon membrane, the diagnostic 400-bp fragment shown by the arrow specifically hybridized with a ^{32}P-labeled *lacZ* DNA probe (data not shown).

liver was previously reported using direct injection (25) or bombardment (26) of DNA plasmids into the organ, injection of vesicle complexes (27) or retroviruses (28) into the portal vein, or injection of DNA complexed with asialoglycoproteins in the blood stream (29, 30). However, none of these reports documented the nature of the cells in which foreign DNA was expressed. Indeed the liver tissue associates many different cell types including phagocytic cells, and most of the liver metabolic and secretory functions are provided by hepatocytes only. Those latter cells should be the preferential target for a therapeutic gene transfer when the restoration of normal functions to a genetically deficient liver or the replacement of a missing diffusible factor is desired. The hepatocyte mitotic index in the steady-state liver is 0.1–0.5% (31, 32). This low turnover suggests that direct *in situ* gene transfer into hepatocytes might be compatible with long-term complementation of genetic deficiencies.

The procedure that we describe here could be technically adapted to human patients. Indeed, human hepatocytes are sensitive to infection with amphotropic murine retroviruses (our unpublished observations), and selective perfusion of liver lobes as well as partial hepatectomy are surgical procedures currently carried out in human patients undergoing treatment for liver tumors (33).

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